Comparison of the Compositions of Phospholipid-Associated Fatty Acids in Wild-Type and Extracellular Matrix Tenascin-X-Deficient Mice

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Tenascin-X (TNX) is a member of the tenascin family of glycoproteins of the extracellular matrix. We previously showed that TNX regulates the synthesis of triglyceride and the composition of triglyceride-associated fatty acids. The aim of the present study was to determine whether TNX controls the synthesis of phospholipids and the composition of phospholipid-associated fatty acids by using TNX-deficient (TNX−/−) mice and TNX-overexpressing fibroblast cell lines. Thin-layer chromatography of total lipids of the skin and sciatic nerves from wild-type and TNX−/− mice revealed that the amounts of major phospholipids, phosphatidylcholine (PC) and phosphatidylethanolamine (PE), in wild-type and TNX−/− mice are not different. Gas chromatography-mass spectrometry showed that the major fatty acid compositions of PC and PE in wild-type and TNX−/− mice are almost the same. Fibroblast cells stably overexpressing TNX also showed almost the same amounts of PC and PE and almost the same fatty acid compositions of PC and PE as those in mock-transfected cells. These results suggest that TNX regulates the amount of triglyceride and the composition of triglyceride-associated fatty acids but not the amount and species of phospholipids or the composition of phospholipid-associated fatty acids.

Key words tenascin-X; phospholipid; triglyceride; fatty acid; phosphatidylcholine; phosphatidylethanolamine

The tenascin family of extracellular matrix (ECM) proteins is composed of the same types of structural domains: a cysteine-rich segment at the amino terminus followed by epidermal growth factor (EGF)-like repeats, fibronectin type III (FNIII)-like repeats, and a fibrinogen-like domain at the carboxy terminus. Six members of the tenascin family have so far been identified: tenascin/cytotactin (tenascin-C, TNC), restictin/J1-160/180 (tenascin-R, TNR), tenascin-X (TNX), tenascin-Y (TNY), tenascin-W (TNW), and the recently identified tenascin-N (TNN).[1—6] However, members of the tenascin family have distinct roles *in vivo*.[7—9]

Some roles of TNX *in vivo* have been determined from results of analyses of TNX-deficient (TNX−/−) mice. TNX−/− mice show progressive skin hyperextensibility as do patients with Ehlers-Danlos syndrome, a heritable connective tissue disorder.[10] This alteration in skin connective tissue is caused by reduced collagen content. Recently, we found that the expression of type VI collagen as well as collagen-associated molecules are affected by TNX deficiency, indicating that TNX is involved in collagen fibrillogenesis.[11] Actually, TNX was shown to bind to type I collagen and modulate the rate and quantity of collagen fibril formation.[12] It had also been shown that tumor invasion and metastasis in TNX−/− mice are promoted by increased levels of activity of matrix metalloproteinases (MMPs), especially MMP-2.[13] This increased level of MMP-2 was mediated through c-Jun N-terminal kinase and protein tyrosine kinase phosphorylation pathways.[14] These findings suggest that TNX participates in the invasion and metastasis of tumor cells.

We have reported that an increased amount of triglyceride and altered composition of triglyceride-associated fatty acids were found in the skin of TNX−/− mice.[15] It was shown that the levels of saturated fatty acids such as palmitoic acid were decreased but that the levels of unsaturated fatty acids such as palmitoleic acid and oleic acid were increased in TNX−/− mice compared with those in wild-type mice. In contrast, overexpressed TNX in fibroblast cell lines resulted in a significant decrease in the amount of triglyceride and altered composition of triglyceride-associated fatty acids, that is, increase in saturated fatty acids and decrease in unsaturated fatty acids compared with those in mock-transfected cells. These results indicate that TNX is involved in the regulation of triglyceride synthesis and in the regulation of composition of triglyceride-associated fatty acids.

We next focused on how TNX deficiency affects the amounts of phospholipids and the composition of phospholipid-associated fatty acids. Phospholipids are the major lipid components of cell membranes. They not only form the bilayers of membranes but also act as reservoirs for the precursors of first and second messengers such as diacylglycerol and inositol 1,4,5-triphosphate. The most abundant species of phospholipids are phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), and sphingomyelin (SM). Changes in the accretion of specific fatty acids in phospholipids have many potential effects on biological processes in membranes, such as hormone binding or responsiveness, and on activities of specific integral catalytic proteins.

In this study, the amounts of phospholipids and the composition of phospholipid-associated fatty acids in TNX-deficient mice were investigated, and it was found that they were not different in wild-type and TNX−/− mice. Possible reasons for alterations in the amount of triglyceride and the composition of triglyceride-associated fatty acids and no changes in phospholipids or phospholipid-associated fatty acids are discussed in this paper.

MATERIALS AND METHODS

Animals TNX−/− mice were created by TNX gene tar-

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geting in murine ES cells as described previously.[13] TNX−/− mice were further established by backcrossing original TNX−/− mice into a congenic line, C57BL/6. Mice were housed at the Department of Animal Experimentation, Hokkaido University Graduate School of Pharmaceutical Sciences. This study was performed in accordance with the Hokkaido University Guide for the Care and Use of Laboratory Animals.

Cell Lines Immortal fibroblasts were established by ninety subcultures of primary wild-type fibroblasts derived from wild-type mice on postnatal day 1 as described in previous papers.[14,15] Briefly, the immortalized fibroblasts were transfected with pSecFTNX-2 plasmid, which encodes a short alternatively spliced form lacking M3 and M15-M22 FNIII repeats,[16,17] and then selected in 400 μg/ml hygromycin-B (Wako, Osaka, Japan). The estimated molecular size of the overexpressed TNX protein is approximately 350 kDa. Cloned FTNX-12 cells, in which TNX is highly expressed, were established.[14,15] As a negative control, an empty vector, pSecTag2/Hygro B, was transfected, and then FTNX-9 cells were established. These cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Nissui, Tokyo) supplemented with 10% fetal bovine serum (FBS) (JRH Biosciences, U.S.A.) at 37 °C in an atmosphere with 5% CO_2.

Phospholipid Analysis Dorsal skin subcutaneous tissues and sciatic nerves were dissected from 2-month-old weight-matched male wild-type C57BL/6 and TNX−/− mice. More than 5×10^7 FTNX-9 and FTNX-12 cells were harvested. Total lipids were extracted from the cells with chloroform/methanol (1 : 1 and 1 : 2), successively. After the extraction, residual precipitates that included proteins were dissolved in 0.5 M NaOH solution, and then protein concentration was determined using a BCA assay kit (Pierce, IL, U.S.A.). Lipid fractions were evaporated to dryness and then redissolved in chloroform/methanol (1 : 1). For phospholipid analysis, lipids with equal amounts adjusted by protein amounts (approximately 0.25 mg protein) were separated by silica gel high-performance thin-layer chromatography (HPTLC) (Merck, Darmstadt, Germany) with chloroform/methanol/aqueous 12 mM $MgCl_2$ (65 : 25 : 4). The quantity of lipid in each band was measured at 500 nm with a dual-wavelength flying spot scanner (CS9300-PC, Shimadzu, Kyoto, Japan). Purified standard PC, PE and SM used for HPTLC analysis were purchased from Matreya Inc. (Pleasant Cap, PA, U.S.A.).

Analysis of Phopholipid-Associated Fatty Acids For analysis of phospholipid-associated fatty acids, especially PC-associated and PE-associated fatty acids, plates were soaked with isopropanol/aqueous 0.2% $CaCl_2$/methanol (40 : 20 : 7) for 20 s after TLC. Then the PC and PE samples on the plates were transferred to PVDF membranes (Millipore, Billerica, MA, U.S.A.) at 180 °C for 50 s. The samples were extracted from the PVDF membranes with chloroform/methanol (1 : 1), and the extracts were dried with N_2 at room temperature. Methyl esterification of fatty acids was performed as follows. Five percent of hydrogen chloride methanol solution (Wako) was added to the dry residue, and transesterification was performed at 95 °C for 2 h. Following the transesterification step, H_2O was added and the methyl esters of fatty acids were extracted with hexane (Merck). The hexane extracts were dried under a stream of N_2 at room temperature, redissolved in 10-μl hexane, and subjected to gas chromatography-mass spectrometry (GC-MS). The GC-MS analysis was carried out on an HP 6890 series gas chromatograph (Hewlett-Packard Co., Palo Alto, CA, U.S.A.) equipped with a 30-m DB-23 (i.d. of 0.25 mm and film thickness of 0.25 μm) (J&W Scientific, Folsom, CA, U.S.A.) and a mass spectrometer JMS-700TZ (JEOL Ltd., Tokyo, Japan). GC-MS analysis of fatty acids was done according to the conditions described previously.[15] Individual components of the fatty acids were identified by comparing with available known standards. The relative proportion of each fatty acid among the sum of six major fatty acids, myristic acid (C14:0), palmitoic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1) and linoleic acid (C18:2), was calculated.

RESULTS

Phospholipids in Subcutaneous Tissues and Peripheral Nerves from TNX−/− Mice and in TNX-Overexpressing Fibroblast Cells We investigated whether species and amounts of phospholipids are affected by TNX deficiency in subcutaneous tissues and sciatic nerves (Fig. 1). TNX is abundantly present in subcutaneous tissues and peripheral nerves.[15,18] It is known that nerve tissue is highly enriched in lipid content, and phospholipids have special structural and functional roles in nerve tissue. Phospholipids were extracted from sciatic nerves of wild-type and TNX−/− mice and then analyzed by HPTLC. The abundant species of phospholipids detected in subcutaneous tissues (Fig. 1A) and sciatic nerves (Fig. 1B) were PC and PE. However, there was no difference between amounts or between the species of phospholipids in both subcutaneous tissues and sciatic nerves from wild-type and TNX−/− mice.

Next, we examined whether overexpression of TNX influences the species and amount of phospholipids in TNX-overexpressing fibloblast FTNX-12 cells compared with those in control empty vector-transfected FTNX-9 cells. Phospholipids were extracted from these cells and then analyzed by HPTLC. As shown in Fig. 2, the species and amount of phospholipids in FTNX-12 cells were almost the same as those in

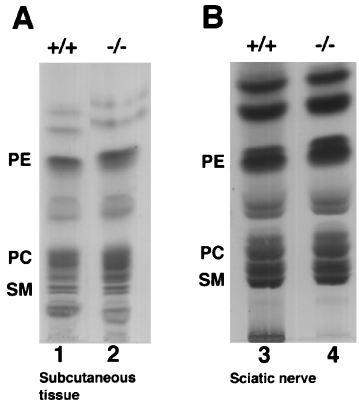

Fig. 1. Analysis of Phospholipids in Subcutaneous Tissues and Sciatic Nerves

Total lipids were extracted from skin subcutaneous tissues (A) and sciatic nerves (B) from wild-type (lanes 1 and 3) and TNX−/− (lanes 2 and 4) mice, separated for phospholipid analysis by HPTLC, and then stained with cupric phosphoric acid. As markers, phosphatidylethanolamine (PE), phosphatidylcholine (PC), and sphingomyelin (SM) are indicated.

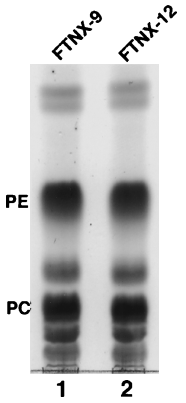

Fig. 2. Analysis of Phospholipids in TNX-Overexpressing Fibroblast Cells

Total lipids were extracted from empty vector-transfected fibroblast (FTNX-9) cells (lane 1) and from TNX-overexpressing fibroblast (FTNX-12) cells (lane 2), separated for phospholipid analysis by HPTLC, and then stained with cupric phosphoric acid. As markers, phosphatidylethanolamine (PE) and phosphatidylcholine (PC) are indicated.

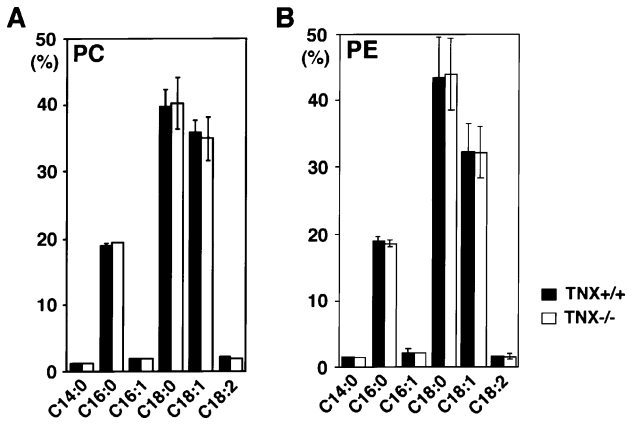

Fig. 3. Analysis by GC-MS of Phosphatidylcholine (PC)- and Phosphatidylethanolamine (PE)-Associated Fatty Acids from Subcutaneous Tissues of Wild-Type and TNX−/− Mice

(A) Relative proportion of each fatty acid content with respective to total content of the six major fatty acids (myristic acid, C14:0; palmitoic acid, C16:0; palmitoleic acid, C16:1; stearic acid, C18:0; oleic acid, C18:1; linoleic acid, C18:2) associated with PC. (B) Relative proportion of each fatty acid content with respective to total content of the six major fatty acids associated with PE. The proportions of the area of each fatty acid in the total area of the six major fatty acids in (A) and (B) were calculated. Black boxes show relative proportions of fatty acids in wild-type mice, and white boxes show those in TNX−/− mice. Data are presented as means±S.E.s of triplicate samples.

FTNX-9 cells, again indicating that TNX does not influence the species and amount of phospholipids.

Phospholipid-Associated Fatty Acids in Subcutaneous Tissues from TNX−/− Mice and in TNX-Overexpressing Fibroblast Cells We examined the compositions of phospholipid-associated fatty acids, especially those of PC-associated and PE-associated fatty acids in subcutaneous tissue of TNX−/− mice compared with those in wild-type mice by GC-MS. The major components of fatty acids associated with PC and PE present in subcutaneous tissues were myristic acid (C14:0), palmitoic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1), and linoleic acid (C18:2). The relative percentages of the individual components among the six major fatty acids associated with PC and PE were shown in Figs. 3A and B, respectively. In both PC-associated fatty acids and PE-associated fatty acids, the relative amounts of each fatty acid were almost the same in wild-type and TNX−/− mice.

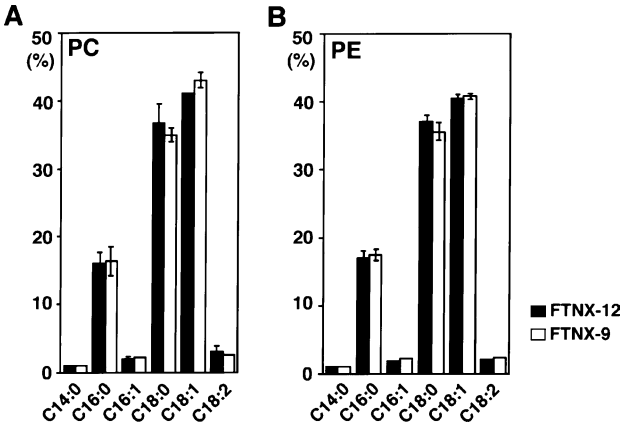

Fig. 4. Analysis by GC-MS of Phosphatidylcholine (PC)- and Phosphatidylethanolamine (PE)-Associated Fatty Acids from TNX-Overexpressing Fibroblast (FTNX-12) Cells Compared with Those from Control Empty Vector-Transfected Fibroblast (FTNX-9) Cells

(A) Relative proportion of each fatty acid content with respective to total content of the six major fatty acids associated with PC. (B) Relative proportion of each fatty acid content with respective to total content of the six major fatty acids associated with PE. The proportions of the area of each fatty acid in the total area of the six major fatty acids in (A) and (B) were calculated. Black boxes show relative proportions of fatty acids in FTNX-12 cells, and white boxes show those in FTNX-9 cells. Data are presented as means±S.E.s of triplicate samples.

The compositions of PC-associated and PE-associated fatty acids in TNX-overexpressing fibroblast FTNX-12 cells were also investigated by GC-MS. The major components of fatty acids associated with PC and PE present in the fibroblast cells were almost the same as those in the subcutaneous tissues. GC-MS analysis showed that there was no difference in the relative amounts of each fatty acid associated with PC (Fig. 4A) and PE (Fig. 4B) in FTNX-12 and empty vector-transfected FTNX-9 cells.

These results indicate that TNX does not influence the compositions of PE-associated and PC-associated fatty acids.

DISCUSSION

In this study, we found that the amounts of phospholipids and the compositions of phospholipid-associated fatty acids, particularly those of PC-associated and PE-associated fatty acids, in the skin of wild-type and that of TNX−/− mice were not different. We also found that overexpression of TNX in fibroblast cells does not influence the amounts of phospholipids or the compositions of PC-associated and PE-associated fatty acids. In contrast, our previous data indicated that the amount of triglyceride was increased, the level of saturated fatty acid was decreased and the levels of unsaturated fatty acids were increased in TNX−/− mice compared with those in wild-type mice. Overexpression of TNX in fibroblast cells gave opposite results.[15]

In TNX−/− mice, increased fatty acid synthesis was correlated with elevated acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) steady-state mRNA levels. It is known that triglyceride and PC are diverted from diacylglycerol (DG) *via* different pathways catalyzed by diacylglycerol *O*-acyltransferase and diacylglycerol choline phosphotransferase, respectively. The pathway of the diversion of DG to triglyceride or PC is dependent on the activity of CTP: phosphocholine cytidyltransferase (CCT).[19] CCT is a rate-

controlling enzyme for PC biosynthesis and plays a key role in the regulation of total phospholipid production, because PC is a precursor to the other two major phospholipids, PE[20] and SM.[21] That is, inhibition of CCT activity diverted newly synthesized DG to triglyceride, whereas forced expression of CCT stimulated PC biosynthesis and reduced triglyceride synthesis. CCT activity governs the partitioning of DG into either the PC or triglyceride pools, thereby controlling both PC and triglyceride biosynthesis. Therefore, CCT activity level may be lower in TNX−/− mice than in wild-type mice. Consequently, even though there are larger amounts of fatty acids in TNX−/− mice, increase in the PC synthesis might not be caused in TNX−/− mice.

At present, the molecular mechanism by which TNX deficiency leads to the alteration of triglyceride-associated fatty acid compositions, not of phospholipid-associated fatty acids, is not clear. However, Cinci *et al.*[22] have shown that the sex hormones affect the fatty acid compositions of phospholipids, triglycerides, and cholesterol esters in the various levels, especially apparent alteration in triglycerides. They speculated that triglycerides are affected by the hormones on fatty acid metabolism better than other lipids since triglycerides quantitatively constitute the highest percentage of body lipids. TNX deficiency also might more affect fatty acid metabolism of triglycerides than phospholipid-associated fatty acids. Furthermore, it is known that fatty acid desaturases introduce a double bond in a specific position of long-chain fatty acids. For example, Δ9-desaturase catalyzes synthesis of oleic acid, a monounsaturated fatty acid, from stearic acid in triglycerides, and is also used for phospholipid.[23] However, if some different desaturases exist for desaturation of triglyceride-associated and phospholipid-associated stearic acids, the different levels of desaturation of stearic acid in triglycerides and phospholipids may be explained in TNX−/− mice.

In conclusion, we have presented evidence that TNX does not regulate phospholipid synthesis or the composition of phospholipid-associated fatty acids. Thus, increase in triglyceride and alteration of the composition of triglyceride-associated fatty acids occur specifically in TNX−/− mice.

Acknowledgments We thank Kiyomi Takaya for her technical assistance. This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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